Mapping of catalytically important residues in the rat L-histidine decarboxylase enzyme using bioinformatic and site-directed mutagenesis approaches

John V. Fleming  
*University of Massachusetts Medical School*

Francisca Sanchez-Jimenez  
Aurelio A. Moya-Garcia

*See next page for additional authors*

Follow this and additional works at: [http://escholarship.umassmed.edu/oapubs](http://escholarship.umassmed.edu/oapubs)

Part of the [Computational Biology Commons](http://escholarship.umassmed.edu/oapubs), and the [Molecular Genetics Commons](http://escholarship.umassmed.edu/oapubs)

Repository Citation


[http://escholarship.umassmed.edu/oapubs/261](http://escholarship.umassmed.edu/oapubs/261)

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in Open Access Articles by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
Mapping of catalytically important residues in the rat L-histidine decarboxylase enzyme using bioinformatic and site-directed mutagenesis approaches

Authors
John V. Fleming, Francisca Sanchez-Jimenez, Aurelio A. Moya-Garcia, Michael R. Langlois, and Timothy C. Wang

Rights and Permissions
Citation: Biochem J. 2004 Apr 15;379(Pt 2):253-61. Link to article on publisher's site

This article is available at eScholarship@UMMS: http://escholarship.umassmed.edu/oapubs/261
Mapping of catalytically important residues in the rat L-histidine decarboxylase enzyme using bioinformatic and site-directed mutagenesis approaches

John V. Flemings†1,2, Francisco Sánchez-Jiménez†1, Aurelio A. Moya-García†, Michael R. Langlois* and Timothy C. Wang*†

*Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01653, U.S.A., †Department of Molecular Biology and Biochemistry, Faculty of Sciences, University of Málaga, Málaga 29071, Spain

INTRODUCTION

Histamine is essential for the differentiation and function of mast cells, the regulation of circadian rhythms and appetite, gastric acid secretion, angiogenesis and the loss of bone in osteoporosis [1–3]. In spite of the importance of these processes, and the clear involvement of histamine in various human pathologies (inflammation-related diseases, neurological disorders, peptic ulcer, etc.), the enzyme responsible for its synthesis in mammals, HDC (L-histidine decarboxylase; EC 4.1.1.1.22), has still not been fully characterized [1]. This is due largely to the low abundance of histamine-producing cells, as well as to the general instability of the enzyme [4–6].

HDC (L-histidine decarboxylase), the enzyme responsible for the catalytic production of histamine from L-histidine, belongs to an evolutionarily conserved family of vitamin B6-dependent enzymes known as the group II decarboxylases. Yet despite the obvious importance of histamine, mammalian HDC enzymes remain poorly characterized at both the biochemical and structural levels. By comparison with the recently described crystal structure of the homologous enzyme L-DOPA decarboxylase, we have been able to identify a number of conserved domains and motifs that are important also for HDC catalysis. This includes residues that were proposed to mediate events within the active site, and HDC proteins carrying mutations in these residues were inactive when expressed in reticulocyte cell lysates reactions.

While the exact cellular mechanisms involved in post-translational processing remain unclear, it has nevertheless been shown that a recombinant version of the enzyme C-terminally truncated to amino acid 516 was fully active when expressed in vitro [14]. In these studies, expression occurred in the absence of significant additional processing. This demonstrated that N-terminal HDC fragments retain all the sequence information required for histidine decarboxylation. An even shorter version, HDC1/477, has more recently been shown to retain activity when expressed in vivo. However, the removal of an additional 5 amino acids (residues 472–477, AANLV) gave an inactive HDC1/472 enzyme [14]. HDC1/477 therefore appears to be the minimal C-terminally truncated unit that encodes all the sequence information required for activity.

Our studies also suggest that a significant change in quartenary structure occurs during catalysis. This involves a protease sensitive loop, and incubating recombinant HDC with an L-histidine substrate analogue altered enzyme structure so that the loop was no longer exposed for tryptic proteolysis. In total, 27 mutant proteins were used to test the proposed importance of 34 different amino acid residues. This is the most extensive mutagenesis study yet to identify catalytically important residues in a mammalian HDC protein sequence and it provides a number of novel insights into the mechanism of histamine biosynthesis.

Key words: L-amino acid decarboxylase, histamine, histidine decarboxylase (HDC), site-directed mutagenesis.

Abbreviations used: HDC, L-histidine decarboxylase; DDC, L-DOPA decarboxylase; GAD, glutamic acid decarboxylase; HA, haemagglutinin; PLP, pyridoxal phosphate; HME, histidine methyl ester.

† These authors contributed equally to this work.
† To whom correspondence should be addressed (e-mail john.fleming@umassmed.edu or timothy.wang@umassmed.edu).
His-274, is located within a conserved hydrophobic domain, but its role in HDC catalysis remains unclear [4]. Other mutations, LIP492/493/494HAS and SKD502/503/504PNS, have also been introduced, but did not decrease catalytic activity [14]. To our knowledge, the four mutations described here are the only ones that have ever been introduced into mammalian HDC protein sequences.

Despite the ongoing problems involved in the crystallization of mammalian HDC enzymes, the importance of histamine and HDC-involving processes requires that attempts should still be made to identify catalytically important residues. It is noteworthy therefore that mammalian HDCs exhibit significant sequence homology with members of the evolutionarily conserved family of dimeric group II l-amino acid decarboxylases. Accordingly, HDC enzymes are hypothesized to share structural and catalytic features with enzymes like mammalian DDC (L-DOPA decarboxylase), and the two mammalian GAD (glutamic acid decarboxylase) isoenzymes GAD-1 (67 kDa) and GAD-2 (65 kDa) [16–20].

Homology with members of the evolutionarily conserved family of dimeric group II l-amino acid decarboxylases. Accordingly, HDC enzymes are hypothesized to share structural and catalytic features with enzymes like mammalian DDC (L-DOPA decarboxylase), and the two mammalian GAD (glutamic acid decarboxylase) isoenzymes GAD-1 (67 kDa) and GAD-2 (65 kDa) [16–20].

Interestingly, it was not possible to get accurate atomic coordinates for amino acids 328–339 of the DDC X-ray crystal structure, indicative of a region of disordered electron density and possibly reflective of a mobile or flexible loop [21].

Recently, an X-ray crystal structure of pig DDC has been described [21], allowing for the positive identification of a number of structural features that are important for the catalytic mechanism. In these studies, it has been noted that two regions in particular, i.e. the region between residues 65 and 115 (DDC region 1) and the region between residues 251 and 312 (DDC region 2), contribute multiple amino acid residues to the active site. Region 1 appears to mediate interactions with the substrate and substrate analogues, while region 2 defines the PLP-binding pocket, and includes residue Lys-303 with which the cofactor forms an internal aldimine in the absence of substrate [21].

Interestingly, it was not possible to get accurate atomic coordinates for amino acids 328–339 of the DDC X-ray crystal structure, indicative of a region of disordered electron density and possibly reflective of a mobile or flexible loop [21]. Evidence that this domain does indeed move during catalysis has come from studies where DDC was proteolytically digested at a trypsin-sensitive site located within the loop. Addition of the substrate analogue L-DOPA methyl ester protected DDC against this proteolysis. This has been taken to reflect a significant structural change that occurs when the active site is occupied, and indicated that the flexible loop had moved so that it was no longer exposed for tryptic digestion [21,22].

In this study we exploit the fact that pig DDC is 51 % identical to the N-terminal catalytic domain of rat HDC, and use the recently described DDC X-ray crystal structure as a starting point with which to predict amino acids that might be of importance for HDC catalysis. We describe 27 separate mutations that were introduced into an active HDC isoform to test specifically these predictions. Our study provides new insights into the biochemical and biophysical properties of HDC, and the mechanism of histamine biosynthesis.

### EXPERIMENTAL

#### Plasmid DNA constructs

The pEP7-HA empty and pEP7-FL empty vector constructs used in this study were generated by cloning double-stranded oligonucleotides, for which the sense strands are shown in Table 1, into the NheI and NotI sites of the pEP-empty vector, which has already been described [5]. These vectors contain CMV (cytomegalovirus) and T7 promoter sequences upstream, and respectively HA (haemagglutinin) and FLAG (FL) tag sequences downstream from the multiple cloning sites. For the pEP-HDC1/516HA and pEP-HDC1/516FL vectors the sense and antisense primers described in Table 1 were used to amplify a fragment from the pEP-HDC2.4 vector [5], which was then cloned into the HindIII/SalI sites of the respective vectors. This fragment corresponded to amino acids Met–1–Glu-516 of the rat HDC protein sequence [14]. Enzyme activities for the tagged HDC1/516HA and HDC1/516FL did not differ significantly from one another or from an untagged HDC1/516 protein (not shown), indicative that the activity associated with the HDC1/516 fragment is independent of C-terminal tags.

The pNET7-empty vector was generated by cloning a double-stranded oligonucleotide, for which the sense strand is shown in Table 1, into the NheI/BamHI sites of the pET11a vector. pNET7-HDC1/516His was generated by cloning a PCR product amplified using the sense and antisense primers shown in Table 1, and the pEP-HDC1/516HA insert as a template, into the BamHI/NotI sites of the pNET7-empty vector.

The pEP-HDC1/516HA, pEP-HDC1/516FL and pNET-HDC1/516His constructs along with a mutant pEP-HDC1/516HA-9G vector described in Table 2 were used as templates to introduce specific mutations using the QuikChange mutagenesis kit (Stratagene). The sense primers used for targeted mutagenesis are shown in Table 2. The successful introduction of mutations was confirmed by partial sequencing.

#### Coupled transcription/translation reactions

*In vitro* transcription/translation reactions were performed using rabbit reticulocyte lysates with 1 µg of test or empty vector plasmids (TNT-Quick kit; Promega). Reactions were supplemented with PLP (0.1 mM). After completion of 10 µl reactions, 30 µl of 0.1 M sodium phosphate buffer (pH 7.4) containing 20 µg/ml cycloheximide was added. Reactions were supplemented with unlabelled or radiolabelled methionine as advised by the manufacturer. Unlabelled reaction products were subsequently used in enzyme assays. Reactions using 35S-radiolabelled methionine...
were always performed in parallel to ensure that differences in enzyme activity were not related to differences in expression levels.

Purification of recombinant proteins

Escherichia coli BL21(DE3) LysS cells, transformed with the pNET7-HDC1/516His or pNET7-HDC1/516His-Y83G plasmids, were grown until the $D_{600}$ value reached 0.4, and were then induced with 1 mM isopropyl-β-D-thiogalactoside for 5 h at 37 °C. Cells were lysed by sonication in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM PLP and 150 mM NaCl (buffer A), and 2 mM imidazole. His-tagged protein was purified by batch purification over Ni$^{2+}$-nitriloacetate beads as advised by the manufacturer (Qiagen), washed three times with buffer A/20 mM imidazole, and eluted with bufferA/80 mM imidazole. Aliquots (500 μl) of partially purified (typically > 60%) by Coomassie Blue staining) extracts were dialysed overnight at 4 °C. Protein concentration was determined by the method of Bradford and 200 μg fractions were used for enzyme assays or gel electrophoresis on denaturing SDS/polyacrylamide gels. In assays to study profiles of tryptic digestion, 200 μg of extract was incubated with 1 mM HME (histidine methyl ester; Sigma) in a 27.5 µl reaction volume at room temperature. After 1 h, 2.5 µl of 10 ng/ml trypsin (Sigma) was added for 15 or 60 min before samples were finally placed on ice.

Assay of HDC activity

Activity values were determined from within the linear range of the activity curve as described previously [5,23] using diluted reticulocyte reaction products or partially purified recombinant protein extracts. Reported enzymic activities are representative of three independent experiments (means ± S.D.). Results were analysed by Student’s t test.

Protein electrophoresis

Samples were fractionated on denaturing 8 % SDS/polyacrylamide gels at 4 °C. Fractionated gels were transferred to PVDF membrane. For radiolabelled samples membranes were dried and exposed directly to autoradiographic film. For non-radiolabelled samples, immunoblotting using an anti-HDC antibody (Accurate Chemical and Scientific) was performed as described previously [14]. All gels shown are representative of at least three independent experiments.

RESULTS

Amino acid residues known to contribute to the pig DDC active site are conserved in the rat HDC protein

Pig DDC, which shares catalytic features with rat HDC [15,24], has recently been crystallized, and domains that are important for catalysis have been identified. Two regions, between residues 63 and 115 (DDC region 1; Figure 1, top panel) and residues 249 and 311 (DDC region 2; Figure 1, middle panel), are particularly important, and a model of the active site that brings together residues from region 1 (Tyr-79, Thr-82 and Ile-101) and region 2 (Asp-271, Asn-300, His-302 and Lys-303) is reproduced in the upper part of Figure 1 (bottom panel). Active-site residues are shown in combination with the PLP cofactor and the L-DOPA substrate analogue carbi-DOPA.
Region #1

<table>
<thead>
<tr>
<th>Pig DDC</th>
<th>Rat HDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>67</td>
</tr>
<tr>
<td>IIMPGVTHW-SAPPFTASYP-PADMLCAG</td>
<td>IIMPGVTHW-CPSYMPH-AYLYT</td>
</tr>
<tr>
<td>TAPELE</td>
<td>TAPELE</td>
</tr>
</tbody>
</table>

Region #2

<table>
<thead>
<tr>
<th>Pig DDC</th>
<th>Rat HDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>249</td>
<td>254</td>
</tr>
<tr>
<td>ESFDNLVEYPI</td>
<td>CAFKDLSEL</td>
</tr>
</tbody>
</table>

A rat HDC1/477 isoform is enzymically active and hence contains all the sequence information required for catalysis. This N-terminal fragment of rat HDC is 51% identical to the pig DDC enzyme, which contains 480 amino acids. Within region 1 described above the two enzymes are 66% identical and in region 2 they are 65% identical. This is shown in Figure 1 (top and middle panels, respectively). Substituting the residues from the DDC active site with the corresponding residues from HDC indicates that 5 of the 8 amino acids that occupy the DDC active site are identical in HDC (63%) including Tyr-83, His-197, Asp-276, Asn-305 and Lys-308. This is shown in the lower half of Figure 1 (bottom panel).

The structure of mammalian HDC has not yet been determined and there is a general lack of information concerning the amino acid residues that are required for catalysis. Based on the model in Figure 1 (bottom panel) residues Asp-276, Asn-305, Ser-307 and...
Figure 2 Mutation of residues predicted to constitute the rat HDC active site

Figure 2 (top panel) also highlights conservation of the His-274 residue (open box). Mutation of this residue has previously been shown to decrease activity, although its proximity to the active site and role in catalysis has been unclear [4]. Taken together these combined analyses suggest a highly conserved role for these residues in mediating PLP-related events in the HDC active site.

To test the importance of residues from this fragment in HDC catalysis a series of mutant HDC1/516 isoforms were generated and expressed in reticulocyte cell lysate reactions. Initially, expressed proteins contained mutations at residues H274Q/V275T/D276R, E288T/L289R/R290D and D300T/S301R. Reactions were tested for enzymic activity and demonstrated that the mutation of residues His, Val and Asp, and of Asp and Ser, significantly decreased enzymic activity, whereas mutation of residues Glu, Leu and Arg, did not (Figure 2, middle panel). It is noteworthy therefore that mutant E288T/L289R/R290D, which had the least effect on enzymic activity, belongs to a region between residues 280 and 295 of HDC that show least identity with the DDC and GAD enzymes (Figure 2, top panel). Subsequently, experiments where amino acid Asp-276, Ser-307 and Lys-308 were individually mutated to glycines confirmed that these specific residues from the HDC active-site model in Figure 1 (bottom panel) are all individually important for catalysis (Table 3).

Another predicted active-site residue, His-197, does not belong to region 2 shown in Figure 1 (middle panel), but is nevertheless predicted to be important for cofactor positioning in the lower part of Figure 1 (bottom panel). By Clustal alignment analysis this residue is also shown to be conserved across other group II decarboxylase enzymes (DDC residue His-192, GAD-1 residue His-291, GAD-2 residue His-282). Mutating this residue in the HDC protein sequence significantly decreased activity of the enzyme (Table 3).

Table 3 Activity of wild-type and mutant proteins expressed in coupled transcription/translation reactions

<table>
<thead>
<tr>
<th>HDC isofrom</th>
<th>Enzymic activity (pmol/µg of DNA per h)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDC1/516HA</td>
<td>9.4 ± 1.8</td>
<td>20</td>
</tr>
<tr>
<td>HDC1/516HA-D276G</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-S307G</td>
<td>0.7 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-K308G</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-H197G</td>
<td>0.8 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-4G</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>HDC1/516HA-2G</td>
<td>4.1 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td>HDC1/516HA-3G</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>HDC1/516HA-9G</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>HDC1/516HA-9G-Y337</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-9G-R339</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-9G-Y337/R339</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-G343 insert</td>
<td>0.8 ± 0.5</td>
<td>3</td>
</tr>
<tr>
<td>HDC1/516HA-Q343G344 insert</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 2 (top panel) also highlights conservation of the His-274 residue (open box). Mutation of this residue has previously been shown to decrease activity, although its proximity to the active site and role in catalysis has been unclear [4]. Taken together these combined analyses suggest a highly conserved role for these residues in mediating PLP-related events in the HDC active site.

To test the importance of residues from this fragment in HDC catalysis a series of mutant HDC1/516 isoforms were generated and expressed in reticulocyte cell lysate reactions. Initially, expressed proteins contained mutations at residues H274Q/V275T/D276R, E288T/L289R/R290D and D300T/S301R. Reactions were tested for enzymic activity and demonstrated that the mutation of residues His, Val and Asp, and of Asp and Ser, significantly decreased enzymic activity, whereas mutation of residues Glu, Leu and Arg, did not (Figure 2, middle panel). It is noteworthy therefore that mutant E288T/L289R/R290D, which had the least effect on enzymic activity, belongs to a region between residues 280 and 295 of HDC that show least identity with the DDC and GAD enzymes (Figure 2, top panel). Subsequently, experiments where amino acid Asp-276, Ser-307 and Lys-308 were individually mutated to glycines confirmed that these specific residues from the HDC active-site model in Figure 1 (bottom panel) are all individually important for catalysis (Table 3).

Another predicted active-site residue, His-197, does not belong to region 2 shown in Figure 1 (middle panel), but is nevertheless predicted to be important for cofactor positioning in the lower part of Figure 1 (bottom panel). By Clustal alignment analysis this residue is also shown to be conserved across other group II decarboxylase enzymes (DDC residue His-192, GAD-1 residue His-291, GAD-2 residue His-282). Mutating this residue in the HDC protein sequence significantly decreased activity of the enzyme (Table 3).

Residues predicted to mediate interactions with the substrate

Based on the alignments and models in Figure 1, amino acids located between residues 67 and 119 of the protein sequence would be expected to contribute to the HDC active site and be of importance for substrate recognition. Ala-86 for example would appear to be involved, and mutation to threonine (the corresponding residue in the DDC protein sequence) inactivated the enzyme (Figure 2, bottom panel). Similarly, our model proposes a role for Tyr-83, and a mutant HDC1/516 isoform.
containing the mutation Y83G was likewise inactive when expressed in reticulocyte cell lysate reactions (Figure 2, bottom panel). This Y83G mutant isoform retained dimerization capabilities, indicating that overall protein structure had not been compromised by this single inactivating mutation (results from stringent co-immunoprecipitations using FLAG- and HA-tagged HDC1/516-Y83G isoforms are not shown). Subsequent experiments performed with partially purified recombinant HDC1/516His-Y83G confirmed in an independent experimental model the effect of this mutation on enzyme activity. On native polyacrylamide gels the HDC1/516His-Y83G mutant migrated at the same size as the wild-type protein, and with the apparent molecular mass of a dimer (results not shown).

**Fragment 330–350 and sensitivity to tryptic digestion**

Previous studies on DDC have identified a domain referred to as the 'flexible loop', whose dynamic properties appear to be important for catalysis and which is a target for proteolytic digestion under control conditions [21,22]. We wondered whether a similar region is located in the HDC enzyme and we generated an active 6 × His-tagged HDC1/516His isoform that was overexpressed and purified from bacteria. Recombinant HDC1/516His was incubated in the presence and absence of 1 ng/ml bovine trypsin for 1 h. As is clear from Figure 3 (top panel), this treatment resulted in the production of a ≈ 36 kDa proteolytic product. A ≈ 36 kDa band, indicative of proteolysis somewhere between amino acid residues 330 and 350 of the rat HDC protein sequence (computer prediction), is also observed in cells transfected to express the full-length HDC isoform [14]. Such proteolysis would correspond exactly to the domain of DDC that is described as the protease-sensitive flexible loop. The two enzymes exhibit 50% identity in this region (Figure 3, middle panel), although it is noteworthy that the DDC protein sequence contains a Gln-Gly insertion (see below).

In DDC, the addition of a substrate analogue (DOPA methyl ester) resulted in a dynamic change in quartenary protein structure so that the protease-sensitive site of the flexible loop was no longer exposed and was therefore protected against tryptic digestion [22]. This indicated that significant structural changes occur when the active site is occupied by the external aldimine moiety during catalysis. To test whether the corresponding element of HDC moves during catalysis, the recombinant HDC1/516His protein was incubated in the presence or absence of HME. This is an analogue of the L-histidine substrate that has previously been shown to block the catalytic reaction after formation of an external aldimine with PLP within the active site [15,25]. Following a 1 h incubation with the substrate analogue, 1 ng/ml trypsin was added for 15 or 60 min time periods. As shown in Figure 3 (bottom panel), incubation with the substrate analogue clearly protected the enzyme against proteolysis, suggestive indeed of a significant change in enzyme structure during the catalytic reaction. Arrows on the right-hand side are indicative of the uncleaved (upper) and cleaved (lower) protein fragments.

According to studies on DDC it is the binding of substrate or substrate analogues within the active site that triggers the detected changes in enzyme structure [22]. It would be anticipated therefore that a mutant form of HDC deficient in substrate binding would not undergo structural changes, and proteolysis at the flexible loop would occur in both the presence and absence of substrate analogue. To test this, and as an independent means of examining the proposed role of tyrosine residue Tyr-83 in substrate binding, we overexpressed and partially purified the recombinant HDC1/516His-Y83G mutant. As shown in Figure 4, treatment with HME did not protect the Y83G mutant enzyme against tryptic digestion and formation of the 36 kDa cleavage product (compare Figure 4 and Figure 3, bottom panel).

**Tyr-337 in the flexible loop of HDC is important for catalysis**

These results suggested that a significant structural change occurs in the trypsin target fragment when substrate analogues enter the...
This mutant retained about 50% activity, which was mutated at RH339/340GG. To test more specifically the role of these residues in catalysis the HDC1/516HA-2G counterpart in HDC is Arg-339/His-340. To test more specifically the roles they might play. For example, it has been proposed that the loop region between residues 331 and 342 is specifically involved in the catalytic mechanism. It could, after all, be an element that simply moves when the active site is occupied, or moves in response to the rearrangement of some other catalytically important domain. To determine whether amino acids in the flexible region are specifically important for catalysis, a mutant isoform referred to as HDC1/516HA-9G was generated. For this isoform amino acids NPIYLRHAN located between residues 334 and 342 within the central region of the loop (see Figure 3B) were collectively mutated to GGGGGGGGG. The HDC1/516HA-9G mutant was inactive (Table 3).

These results confirmed the general involvement of residues from the 331–344 region in HDC catalysis, but provided little insight into the specific residues that were important or the roles they might play. For example, it has been proposed that tryptic digestion of DDC occurs within the flexible loop between residues Lys-334 and His-335 (see Figure 3, middle panel). The counterpart in HDC is Arg-339/His-340. To test more specifically the role of these residues in catalysis the HDC1/516HA-2G mutant, which was mutated at RH339/340GG, was generated. This mutant retained about 50% activity, suggesting that these residues are less important for catalysis (Table 3).

On the other hand, Tyr-337 in the flexible loop is highly conserved in a number of PLP-dependent enzymes [21,22]. To test the importance of the Tyr-337 residue in HDC catalysis a mutant HDC1/516HA-1G isoform was generated with a glycine residue at position 337. This completely abolished HDC activity (Table 3) and identified residue Tyr-337 as critical for catalysis. A mutant protein carrying mutations at positions YRH337/339/340GG (HDC1/516HA-3G) was similarly inactive (Table 3).

Other residues in this region are also required, however, and re-introducing Tyr-337, Arg-339 and Tyr-337/Arg-339 sequentially back into the inactive HDC1/516HA-9G mutant protein (where all nine residues between 334 and 342 were mutated to glycine) did not restore catalytic activity (HDC1/516HA-9G-337Y, HDC1/516HA-9G-339R and HDC1/516HA-9G-337Y339R, Table 3).

One of the more interesting elements of this structural loop feature, and a possible factor in the substrate-specific differences between DDC and HDC, is the fact that DDC has a 2-amino-acid insertion (Gln-Gly) in this domain (Figure 3, middle panel). It is not clear how important these residues are for DDC catalysis, but we can report here that sequentially introducing Gln and Gln-Gly residues into the corresponding site of HDC decreased (by $\approx 90\%$) and abolished enzymic activity respectively (HDC1/516HA-Q343 insert and HDC1/516HA-Q343G344 insert, Table 3).

**Cysteine residues located in regions 1 and 2 are important for HDC catalysis**

In the DDC enzyme Cys-111 has been proposed to play an important structural role [26], and in the DDC crystal structure this residue is sufficiently close to residue Cys-100 to permit formation of a disulphide bridge [21]. Nevertheless, it has been argued that if such a bond were to be formed it would probably distort the active site and lead to inactivation of the enzyme [21]. In the case of HDC, enzyme preparations under oxidative conditions lead to aggregation of the enzyme. Experimental conditions can be created therefore where disulphide bridges are indeed formed within HDC; however, these aggregates are catalytically inactive [15,27].

While disulphide bridges are unlikely to be a feature of the normal catalytically competent HDC enzyme, cysteine residues Cys-101 and Cys-115, along with Cys-254, Cys-266 and Cys-316, are all conserved between the HDC and DDC enzymes and all are located within what were described in Figure 1 (top and middle panels) as the catalytically important regions 1 and 2. These residues might consequentially be expected to influence the topology of the active site in their own right. To test the importance of these residues in HDC catalysis, all nine cysteine residues in the HDC1/516 protein fragment were mutated to serines. Mutant proteins were expressed in reticulocyte cell lysate reactions. In no case was activity completely abolished, arguing again against an essential role for cysteine residues and hence disulphide bridges (Figure 5). Nevertheless, the activities of proteins mutated at residues Cys-104, Cys-115, Cys-254 and Cys-316 were all significantly decreased relative to the control wild-type enzyme (which had activity levels of $10.9 \pm 1.7 \text{ pmol/µg}$ of DNA per h, $n = 3$). While some cysteine residues are clearly of importance for catalysis therefore, we believe that this is likely to be reflective of the proximity of these residues to the proposed active site. Interestingly, one Cys to Ser mutation at residue 421 actually showed a slight but reproducible increase in HDC activity. This residue is not predicted to form part of the active site and additional studies will be required to fully address the role of this residue in catalysis.

**DISCUSSION**

Here we set out to identify amino acids residues and structural features of the mammalian HDC enzyme that are of importance for histamine biosynthesis. From the outset therefore we were particularly interested in identifying elements that might mediate interactions with the substrate and cofactor, as well as structural features like disulphide bridges or movable elements that might be of importance for maintaining HDC structure and function. Sequence comparison with other group II decarboxylase enzymes allowed us to identify residues in the HDC protein sequence that might have some of these roles, and experimental studies were undertaken to test our proposals. These relied mainly on a reticulocyte cell lysate reaction model, which has been used successfully in the past to study structure/function relationships in a number of different L-amino acid decarboxylase enzymes.
We also identified a trypsin-sensitive site that belongs to the flexible loop domain of HDC, and demonstrate that it becomes protected against proteolysis once the substrate analogue HME enters the active site. While this loop domain appears to be a feature of many PLP-dependent enzymes, the studies described here are the first to confirm the existence of such a region in HDC. For example, in our model we identified a number of residues that we predict to mediate substrate-specific interactions, including Tyr-83 and Ala-86, and mutating these residues certainly decreased activity. For the Tyr-83 mutant in particular the addition of HME did not protect against tryptic proteolysis. In co-immunoprecipitations and under native fractionation conditions this isoform retained dimerization properties, arguing against a widespread disruption of tertiary structure (results not shown). Instead, our results are supportive of a more specific role in substrate interactions, and suggest that formation of the external aldimine moiety within the active site is indeed the molecular trigger for dynamic structural changes. While crystallization will be required to confirm a role for residue Tyr-83 in substrate recognition, the results reported here allow us to speculate nevertheless on the importance of this residue as a direct or indirect link with structural changes in the flexible loop domain. It is important to remember that the co-ordinates for flexible loop residues in the DDC protein crystal remain unresolved, and even when the HDC enzyme eventually gets crystallized it remains likely that disordered electron density in this region, such as that observed for the corresponding region crystallized DDC, will obscure the exact molecular basis for these changes.

Ser-307, which is also one of our predicted active-site residues, is also worthy of special mention. In mammalian DDC, this residue is substituted by His-302. Bertoldi et al. [31] have reported that a single H302Q replacement in rat DDC changes the spectral properties of the internal aldimine, and the catalytic constant for DOPA decarboxylation is reduced by more than two orders of magnitude. Human GAD-1 and GAD-2 also have histidine residues at this position (Clustal alignment; residues His-404 and His-395 respectively). It could be suspected therefore that the natural His/Ser substitution is partially responsible for the much slower decarboxylation reaction carried out by mammalian HDCs; indeed, it has previously been noted that this parameter is several orders of magnitude slower in mammalian DDCs; indeed, it has previously been noted that this parameter is several orders of magnitude slower than that of homologous enzymes [9,15,32]. It is also noteworthy that the prokaryotic HDC enzyme, which is much more efficient than the mammalian enzyme, also has a histidine residue at this position. More efforts are needed however to fully characterize the role of this residue in the specificity and efficiency of group II decarboxylases.

Supported by NIH grant DK48077 to T.C.W. and grants SAF2002-02586 (MCYT, Spain) and Red Española de Mastocitosis (REMA; ISCIII, Spain) to F.S.-J. A.A.M.-G. was funded by Fundación Ramón Areces and Junta de Andalucía (CIV-267). We thank D. G. Lambright and T.M. Wac for useful discussions.

REFERENCES


© 2004 Biochemical Society
Catalytically important residues in mammalian histidine decarboxylase

Received 3 October 2003; accepted 15 January 2004
Published as BJ Immediate Publication 30 January 2004, DOI 10.1042/BJ20031525


